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Short communication

Profilin 3 genetic architecture in glioma formalin fixed paraffin embedded (FFPE) archive

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ABSTRACT

Pfn3 is an intron-less gene, encoding actin binding protein that affects structure of cytoskeleton. Although, Pfn3 is mentioned in Allen Brain Atlas and in adult and prenatal Human Brain Tissue Gene Expression Profiles dataset, however, no report on brain and/or brain tumor associated Pfn3 nucleotide sequences are available in the databases. Moreover, *pfn3* and *pfn4* are always considered as testicular specific genes. The current study explored transcriptional expression profile and genetic architecture of *pfn3* in a cohort of fifty formalin fixed paraffin embedded (FFPE) human glioma archive tissues. Results of designed study highlighted the significant dysregulated transcriptional pattern of *pfn3*. Molecular similarity index indicated 97% in nucleotide and 93% homology in protein sequences (with clear differences in nine amino acid residues). Thus, molecular variations in the *pfn3* may be corelated with the malignancy of brain tumors, as previously, *pfn1* and *pfn2* were reported as tumor suppressor genes in other types of cancer.

1. Introduction

Pfn family members are cytoskeleton actin binding molecules and regarded as important in development and maturation of brain specifically in the neuronal migration. All four *pfn* isoforms (Huse and Holland, 2010; ShahidMahmood et al., 2016; Central nervous system cancers, 2014; Mamelak and Jacoby, 2007) are encoded by different genes and with different spliced variants of mRNA (Blanchoin et al., 2014; Witke et al., 2001). Predominantly, Pfn1directly affects the structure of the cytoskeleton and plays a significant role in actin dynamics and polymerization (Witke et al., 2001). Pfn2 has been reported as ubiquitous actin monomer-binding protein and regulates actin polymerization in response to extracellular signals (Witke et al., 1998). Despite of known importance of Pfn1 and 2, the molecular involvement of Pfn3 in brain development and processes is an understudied domain' that is limited to its role in the spermatogenesis. However, contrary to other family members, it interacts with pfn3 and binds to phosphatidylinositol phosphatidylinositol 4-phosphate, phosphatidylinositol 3-phosphate 4,5-bisphosphate and phosphatidic acid (Suetsugu et al., 1998).

In the brain pfn1 and Pfn2 connect the actin cytoskeleton and endocytic membrane flow, directing actin assembly to discrete membrane domains (Witke et al., 2001). Moreover, Pfn1 has greater affinity for phosphatidylinositol biphosphate (PIP_2) compared to Pfn2. During actin polymerization PIP_2 competes with pfn1 for binding to poly-L-proline. In general, mutated pfn transcripts resulted in impaired actin polymerization and assembly which resulted in aberrant signaling and may led to the development of tumorigenesis (Lambrechts et al., 1997). The aberrant phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT signaling is recurring theme for numerous types of cancers including brain cancer. PI3K signaling is initiated by the extracellular growth factors that binds to the tyrosine kinase receptor causes the

Abbreviations: EGF, Epithelial growth factor; ABP, Actin binding protein; PFN, Profilin; PIP, Phosphatidylinositol 4,5- bisphosphate; DNA, Deoxyribonucleic Acid; mRNA, Messenger Ribinucleic Acid; cDNA, Complementary Deoxyribonucleic Acid; PCR, Polymerase Chain Reaction; WHO, World Health Organization; HGG, High Grade Glioma; GBM, Glioblastoma multiforme; q RT-PCR, Quantitative Real time Polymerase chain reaction; IP3, Inositolephosphate 3; FFPE, Formalin fixed paraffin embedded.

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dimerization of the receptor, as a result PI3K phosphorylates PIP_2 to PIP_3. PTEN (phosphatase and tensin homolog) antagonizes the activity of PIP3 by reverting it back to PIP3. Activated PIP3 and PI3K recruits AKT, and similarly PDK1 (pyruvate Dehydrogenase Kinase 1) completely activates AKT by phosphorylating it. Activated AKT translocate to cytoplasm and nucleus. The activated AKT inactivates FOXO (a protein/ factor that hinders cell proliferation), inhibits GSK3β (Glycogen synthase kinase 3ß, involved in metabolism) and directly activates MDM2 (mouse double minute 2 homolog) inhibiting p53 (tumor protein p53) all leading towards the development of tumorigenesis (Das et al., 2009; Abedalthagafi et al., 2016).

In the last decades, molecular studies and analysis of diseases results in better diagnosis and prognosis. Although the role of Pfn, AKT, PI3K and PTEN are well studied in breast cancer and other types of cancers; however, the specific functional correlation of Pfn protein isoforms with brain tumor/cancer still needs to be investigated. In this connection, present study aimed to study transcriptional pattern *pfn* isoforms (Huse and Holland, 2010; ShahidMahmood et al., 2016; Central nervous system cancers, 2014; Mamelak and Jacoby, 2007) and explored the genetic architecture of *pfn3* encoding gene in comparison of other *pfn* isoforms.

2. Materials and methods

2.1. Ethical statement

The experimental procedures were carried out according to guidelines of ethical review committee (ERC) of School of Life Sciences, Forman Christian College (A Chartered University) approved by the institutional review board of Forman Christian College (A Chartered University), Lahore, Pakistan.

2.2. Glioma Tissue extraction and cDNA library synthesis

A cohort of 207 formalin fixed paraffin embedded (FFPE) brain tumor tissues were retrieved in the period of last two years from General Hospital, Lahore archive section. All tumor tissues were surgically isolated for biopsy purpose and histopathology reports of all the samples were collected and analyzed. FFPE samples were kept in sealed sterile plastic bags and stored at 4 °C. The samples were classified into different types according to histopathology reports, patient's history and graded according to WHO standards. Samples information, classification, grading and supplementary information such as age, gender, date of surgery and site of biopsy were obtained and arranged accordingly. FFPE tissues (–100 thickness) were de-paraffinized by xylene {600 μl), to dissolve paraffine, for three times followed to a washing with absolute ethanol. Soft, deparaffinized glioma tissues were finally preserved in 30% ethanol and and total RNA was extracted by modified TRIzol method (MRI, Catalog # RT111) and dissolved in 20 μl of nuclease free water. RNA concentration was quantified using Nanodrop (Thermo Scientific 2000c spectrophotometer) (Ma, 2012; Abramovitz et al., 2008). The quality of RNA was accessed on 1% agarose gel using gell documentation system (Gel-Doc-It 310 imaging system). Complementary DNA (cDNA) was synthesized using total RNA (1 μg) by reverse transcriptase PCR (RT-PCR) using Thermo Scientific kit (Catalog# K1622) in accordance with the protocol described by the manufacturer.

2.3. Primer synthesis

Gene specific primers of complete transcripts and quantitative/ real-time (qRT-PCR) for human pfn isoforms; pfn1, pfn2, pfn3 and pfn4 were designed by using reference sequence from National Centre for Biotechnology Information (NCBI). Accession number NM_005022.3 (*pfn1*), NM_053024.3 (*pfn2*), NM_001029886.2 (*pfn3*), and NM_199346.2 *(pfn4*). Primers were design by online available software "GeneScript", with primers lengths of 19–22 bps, specifically for qPCR, expected product kept 100-200bps, GC content of 45–55% and melting temperature (Tm) around 50–60 °C.

2.4. Quantitative transcriptional analysis

Syber Green based qRT- PCR was conducted to analyze the transcriptional profile of pfn isoforms (*pfn1, pfn2, pfn3* and *pfn4*). The reaction mixture comprised of Syber Green mix (Thermo Scientific, Catalog # K0221), 1 μl of cDNA, forward and reverse gene primers. GAPDH was used as housekeeping gene. For relative quantification, non-tumor tissues or normal appearing tissues (NATs) were used as calibrators. The specificity of amplicon was assessed by melt curve analysis. The cycling conditions used for qPCR were: 94 °C for 5 min, 50 cycles of 94 °C for 45 sec, 53 °C for 45 sec, 72 °C for 45 sec, and 72 °C for 7 min; melt curve conditions 65 °C for 30sec, 95 °C for 45 sec, and hold on 25 °C for 20 min. Relative method of quantification was chosen for the transcriptional analysis of *pfn* isoforms. ΔΔCt method was used to determine fold change in the targeted gene expression (Livak, 1997).

2.5. Statistical analysis

Data of qRT-PCR was analyzed by paired sample *t*-test comparing the significant mean fold change in pfn isoforms encoding genes (*pfn1, pfn2, pfn3* and *pfn4*) and calibrator with reference to housekeeping gene GAPDH in patient samples. The association between transcriptional levels of pfn isoforms was analyzed by Pearson correlation. Association of clinical variables and expressional data was analyzed by Kruskal Wallis test. For all the tests a p-value ≤ 0.05 was considered significant.

2.6. Sanger sequencing of pfn transcripts

Full length transcript of four isoforms of *pfn* were amplified and sequenced by Sanger sequencing using commercial services (Europhean, Germany).

3. Results

3.1. Types of brain tumor and gene

3.1.1. Expression of pfn3 in comparison of pfn1, 2 4

Histopathology analysis of 207 brain tumor tissues declared 34.30% tissues were glioma and malignant, however 66.70% tumors were meningioma and benign in nature (Fig. 1A).

The transcriptional levels of *pfn* isoforms (*pfn1, pfn2, pfn3 and pfn4*) in glioma tissues and calibrator were analyzed and compared with GPDH. Significant fold change differences were noticed for each *pfn* isoform p = 0.050, 0.047, 0.010 and 0.038 respectively. *pfn1* showed 22% upregulation and 58% downregulation whereas the no change in expression was observed in 20% samples. 20% of the samples showed upregulation whereas 80% showed downregulation for *pfn2. pfn3* indicated an upregulation in 24% and downregulation in 60% samples whereas 16% samples depicted no change in expression. For *pfn4* 18% samples were seen to be upregulated whereas 82% were downregulated (Fig. 1B).

The association of transcriptional levels of all the four *pfn* isoforms were analyzed through Pearson-correlation test which indicated that expression of *pfn3* and *pfn4* were significantly correlated (p-value = 0.021) whereas no significant correlation of pfn1 was observed with pfn2, *pfn3* and *pfn4* (p-value = 0.295, 0.543 and 0.155 respectively). Furthermore, *pfn2* also showed no significant correlation with pfn3 and pfn4 (p-value = 0.573 and 0.900 respectively).

3.2. Amplification of pfn isoforms

The nucleotide sequence of *pfn1*, 2 and 4 complete transcripts was found 99.9% identical to reference sequences. Interestingly, promising

A

HISTOLOGY	PERCENTAGE
Meningioma	30.9%
Astrocytoma	30.9%
Schwannoma	16.4%
High grade gliomas	1.4%
Pitutary adenoma	1.4%
Craniophryngioma	2.4%
Mixed glial tumor	1.0%
Glioblastoma multiforme	6.8%
Oligidendroglioma	4.8%
Hemangioblastoma	0.5%
Medulloblastoma	1.0%
Ependymoma	0.5%
Chordoma	0.5%
Adenocarcinomma	1.4%

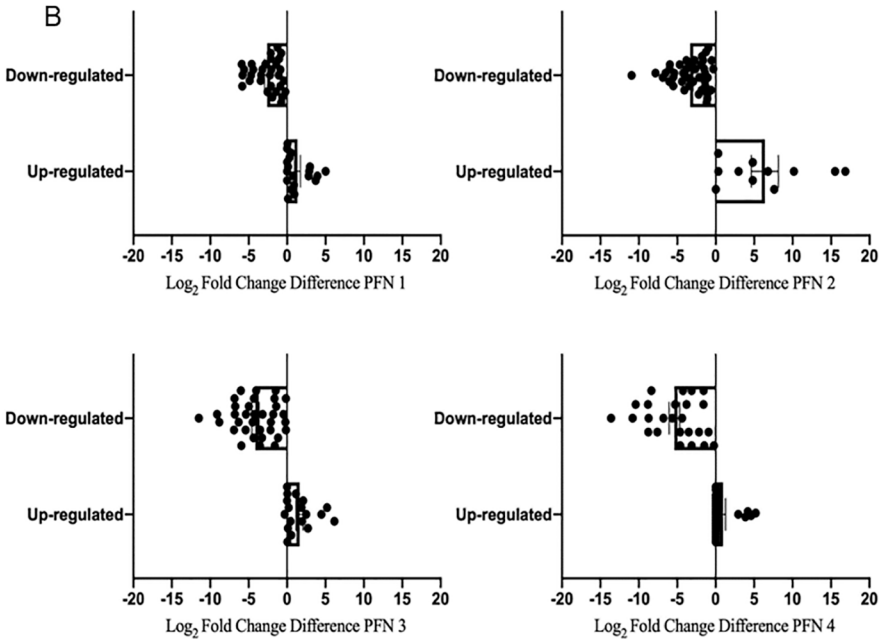

Fig. 1. A. Histological type and percentages of brain tumor. B. Transcriptional expression of *pfn* isoforms () in human gliomas tumor.

nucleotide differences were noticed in *pfn3* transcript (Gen Bank accession number: MT036395) along with 97% homology to the previously known testicular *pfn3* transcript (Gen Bank accession number: NM_001029886.3). Although, the expression of Pfn3 in brain relative to the other tissues, is mentioned in Allen Brain Atlas and in adult and prenatal Human Brain Tissue Gene Expression Profiles dataset, however, nucleotide sequences of *pfn3* were deposited only from testicular source and no brain associated *pfn3* transcript sequence was available in the databases. Moreover, the genetic differences of *pfn3* reflected the significant variability in the predicted protein sequence (93% homology with NP_001025057.1) respectively (Fig. 2).

4. Discussion

Cytoskeletal proteins act as effector target and modulator of signal transduction that contributes to numerous cellular functions and behaviors (Afghani, 0000). The pfn family is one of the cytoskeletal actin binding proteins which are actively expressed in brain cells (Blanchoin et al., 2014). Current study explored the *pfn* gene's involvement in various pathological graded human gliomas tissues.

The demographic analysis of histopathological data provided significant clinical information about the brain tumor and predicted the functional relationship between demographic parameters and tumorigenesis. Among the cohort of 207 brain tumor cases meningioma and

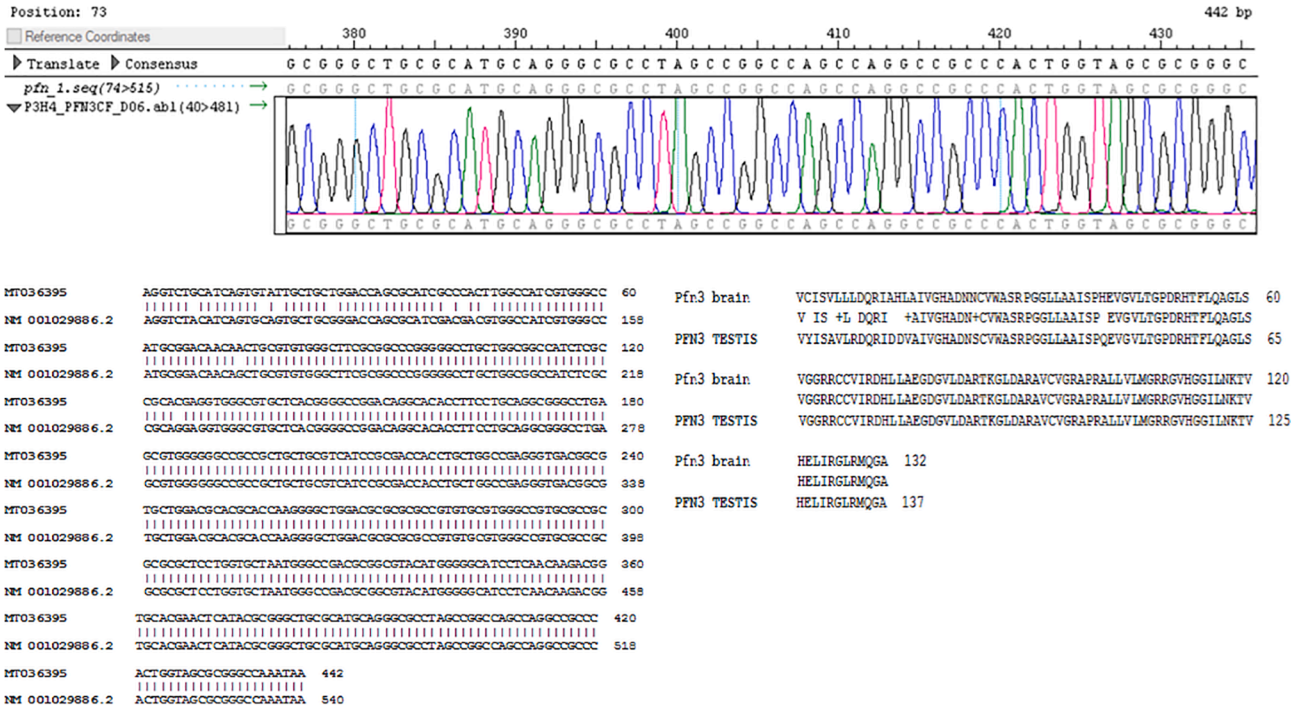

Fig. 2. A. Amplified gene of human *pfn3*, B. Alignment of *pfn3* Nucleotide sequence, C. Predicted Protein sequence of Pfn3 from human glioma tissues.

astrocytoma comprised 30.9% whereas chordoma, ependymoma and hemangioblastoma represented only 0.5% (Fig. 1A). Likewise, meningioma, schwannoma, chraniopharyngioma and pituitary adenoma were grade I tumors. Oligodendrogliomas were grade II and grade III tumors. Whereas most of the adenocarcinoma and medulloblastoma were grade III tumors and all the glioblastoma multiforme were grade IV tumors. Similarly, astrocytoma were high grade gliomas. Furthermore, we observed similar significant ($p = 0.000$) association between tumor grading and histology as previously reported by @@Nomikos et al., (2014) (Nomikos et al., 2014) and that according to WHO standards malignant tumors are classified as high-grade tumors. Meningioma and schwannoma tumors are known as benign, but astrocytoma has almost equal cases of malignant and benign tumors, all the pituitary adenoma and chraniopharyngioma are also known as benign whereas glioblastoma multiforme and oligodendroglioma tumors are typically malignant tumors.

RNA being highly sensitive is prone to degradation and in only present in enormous quantities in fresh samples rather than FFPE samples. The RNA quantification of glioma samples showed considerable results as reported by Haque et al. (2007) and Imboden et al. (1993) that despite extensive RNA degradation most of the FFPE samples give valid reproducible results.

Transcriptional analysis of *pfn* isoforms (*pfn1,* 2, 3 and 4) in glioma tissues and calibrator (NATs; normal appearing tissues adjacent to tumor) exhibit comparable expression with reference to GAPDH as a housekeeping gene with a significant difference in fold change with a p-value 0.052, 0.047, 0.010 and 0.038 respectively (Valente et al., 2009) suggest GAPDH as a suitable housekeeping reference gene for studies on glioblastoma gene expression as it shows considerable difference in expression levels of tumor and controls. The transcriptional expression of the *pfn* indicates significant downregulation of all the four *pfn* isoforms in the present study. Out of the fifty glioma samples twenty-nine samples with *pfn1*, forty samples with *pfn2*, thirty-eight samples with *pfn3* and twenty-eight samples with *pfn4* showed downregulation (lower expression) whereas as eleven with *pfn1*, eleven with *pfn2*, twelve samples with *pfn3* and five with *pfn4* 4 showed upregulation (higher expression). The findings of the study are in accordance with the studies conducted by Janke et al. (2000), Das et al. (2009) and Wittenmayer et al. (2004) suggesting *pfn* to depict lower expression levels linked to tumor state of cancers and therefore indicate downregulation rendering *pfn* as a tumor antioncogene which otherwise positively regulates brain signaling pathways but induces aberrations when downregulated. The present study therefore inferences that *pfn* is a tumor suppressor as its downregulation is associated to tumor state of brain cancer.

The sequence analysis of *pfn2* further confirms the finding that *pfn 1 & 2* are brain specific (Witke et al., 1998, 2001; Gieselmann et al., 1996; Lambrechts et al., 1997). However, sequence analysis of human *pfn3* indicate 97% homology with testicular origin *pfn3* and till date known as testis specific and because of its functional involvement in spermatogenesis (Braun et al., 2002).

The findings of the study suggested a significant association to the PI3K-AKT pathway, indicating downregulation of *pfn* in breast cancer is associated with inactivation of AKT which results in aberration of the pathway and leads to tumorigenesis. Similar association applies to expression of *pfn* in brain tumor which is also observed to be downregulated. Another linkage that supports the findings of the study is the binding of Pfn to PIP2 and PIP3 (Blanchoin et al., 2014; Ridley, 2011; Lambrechts et al., 1997; Krishnan and Moens, 2009; Goldschmidt-Clermont et al., 1990). On conclusionary note the study reveals that expressional studies with FFPE tissues can give reliable and comparable results. The study also identified *pfn3* encoding gene for the first time in human brain and indicates that downregulation of *pfn* isoforms in human glioma indicates that these genes can be regarded as tumor suppressor genes and *pfn* thus can be added to pathological view of glioma.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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